Department of Clinical Veterinary Sciences, Section of Surgery

RADIATION SYNOVECTOMY WITH HOLMIUM-166 FERRIC HYDROXIDE MACROAGGREGATE

Experimental study in rabbits and horses

by
Olli Mäkelä

ACADEMIC DISSERTATION

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Supervised by:

Professor Riitta-Mari Tulamo
Department of Clinical Veterinary Medicine, Section of Surgery
Faculty of Veterinary Medicine, University of Helsinki

Professor Antti Sukura
Department of Basic Veterinary Sciences, Section of Pathology
Faculty of Veterinary Medicine, University of Helsinki

Professor Heikki Helminen
Department of Anatomy
Faculty of Medicine, University of Kuopio

Assistant Professor Mikko Lammi
Department of Anatomy
Faculty of Medicine, University of Kuopio

Reviewed by:

Docent Martti Hannelin
The Central Hospital of Etelä-Saimaa
Lappeenranta

Professor Elwyn Firth
Institute of Veterinary, Animal, and Biomedical Sciences
Massey University, New Zealand

Opponent:

Professor Sheila Laverty
Department of Clinical Sciences
Faculty of Veterinary Medicine
University of Montreal, Canada

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ABSTRACT

Rheumatoid arthritis (RA) is an inflammatory disorder characterized by synovial cell proliferation and microvascular injury. RA often leads to permanent articular cartilage damage because the inflamed synovium is an abundant source of cytokines and enzymes harmful to the articular cartilage. If repeated intra-articular corticosteroid treatments fail to control synovial inflammation, surgical, chemical, and radiation synovectomy (RSYN) with different radioactive preparations have been used to arrest the inflammation and the progression of cartilage degeneration. In this experimental study, the normal joints of rabbits and horses were investigated to assess the effects of RSYN with a relatively new radiopharmaceutical, holmium-166 ferric hydroxide macroaggregate (^{166}Ho FHMA), to determine whether it would be a suitable candidate for therapeutic use in humans, and possibly also in horses.

The treated joints were injected with radioactive ^{166}Ho FHMA and the control joints with nonradioactive ^{165}Ho FHMA. Both in rabbits and horses, radioactive ^{166}Ho FHMA markedly suppressed the synovial lining hyperplasia compared with control joints. In addition, multifocal necrosis of the synovial lining, neovascularization, and eventually subsynovial fibrosis were seen in the joints treated with ^{166}Ho FHMA. In rabbits, the pattern of multifocal synovial necrosis as well as the intra-articular distribution of radioactivity in the autoradiography images suggested an uneven distribution of ^{166}Ho FHMA within the joint. Autoradiography analysis of lapine knees showed some leakage of radioactivity 3 days postinjection. In horses, ^{166}Ho FHMA treatment caused reactive synovitis in fetlock joints. A large dose of strong beta-emitter ^{166}Ho resulted in excessive radiation injury and local pain. Joint fluid analysis of horses showed very little residual radioactivity 5 days after RSYN; ^{166}Ho FHMA had probably been effectively cleared from the synovial fluid by the synovium. Scintigraphic examination revealed no extra-articular leakage of radioactivity. Increased synovial fluid protein levels after RSYN indicated radiation-induced soft-tissue injury and leakage of plasma into the synovial fluid from the vasculature. Synovial restoration in rabbit knees was evident 2 months after ^{166}Ho FHMA, whereas equine synovium had regenerated poorly.

The composition and morphology of adult articular cartilage in both species were not markedly affected by ^{166}Ho FHMA treatment. Young rabbit cartilage showed mild radiation-induced fibrillation of the cartilage surface after ^{166}Ho FHMA treatment but no further breakdown of the collagen framework during the 1-year follow-up. Active ^{35}S sulfate incorporation indicated normal chondrocyte metabolism in all zones of the cartilage. Only transient irradiation-induced derangement of matrix production was seen in young rabbits, and transient downregulation of collagen synthesis in horses.

Overall, suppression of synovial hyperplasia, little extra-articular leakage, and only minor effects on articular cartilage suggest that RSYN can be used in clinical management of arthritis in man and horses provided that untoward side-effects are avoided.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original articles referred to in the text by Roman numerals I-IV (reprinted with the permission of the copyright holders).

I. Mäkelä O, Penttilä P, Kolehmainen E, Sukura A, Sankari S, Tulamo R-M. Experimental radiation synovectomy in rabbit knee with holmium-166 ferric hydroxide macroaggregate. *Nuclear Medicine and Biology 2002; 29: 593-598.*

II. Mäkelä OT, Lammi MJ, Uusitalo H, Hyttinen MM, Vuorio E, Helminen HJ, Tulamo R-M. Analysis of lapine cartilage matrix after radiosynovectomy with holmium-166 ferric hydroxide macroaggregate. *Annals of the Rheumatic Diseases 2003; 62: 43-49.*

III. Mäkelä O, Sukura A, Penttilä P, Hiltunen J, Tulamo R-M. Radiation synovectomy with holmium-166 ferric hydroxide macroaggregate in equine metacarpophalangeal and metatarsophalangeal joints. *Veterinary Surgery 2003; 32: 402-409.*

IV. Mäkelä OT, Lammi MJ, Uusitalo H, Viitanen M, Hyttinen MM, Jurvelin JS, Vuorio E, Helminen HJ, Tulamo R-M. Effect of radiosynovectomy with holmium-166 ferric hydroxide macroaggregate on adult equine cartilage. *The Journal of Rheumatology 2004; 31: 321-328.*

ABBREVIATIONS

ADAMTS	adamalysin subfamily of metalloproteinases
AIA	antigen-induced arthritis
^{198}Au	gold-198
cDNA	complementary deoxyribonucleic acid
dCTP	deoxycytidinetriphosphate
^{165}Dy	dysprosium-165
EDTA	ethylenediaminetetraacetic acid
^{169}Er	erbium-169
FHMA	ferric hydroxide macroaggregate
GAG	glycosaminoglycan
HE	hematoxylin and eosin
^{166}Ho	holmium-166
IL-1	interleukin-1
MCP	metacarpophalangeal joint
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MTP	metatarsophalangeal joint
NaCl	sodium chloride
NaHA	sodium hyaluronate
NF-κB	nuclear factor kappa B
NSAID	nonsteroidal anti-inflammatory drug
OA	osteoarthritis
^{32}P	phosphorus-32
PCR	polymerase chain reaction
PGE_2	E-series prostaglandin 2
PSGAG	polysulfated glycosaminoglycan
RA	rheumatoid arthritis
^{186}Re	rhenium-186
RNA	ribonucleic acid
rRNA	ribosomal RNA
RSYN	radiation synovectomy
RT	reverse transcription
^{35}S	sulfur-35
SDS	sodium dodecyl sulfate
SLRP	small leucine-rich proteoglycan
^{153}Sm M	samarium-153 hydroxyapatite microsphere
SSC	standard saline citrate
TNF-α	tumor necrosis factor alpha
^{90}Y	yttrium-90

INTRODUCTION

In human rheumatoid arthritis (RA), when medical treatment fails, surgical, chemical, and radiation synovectomy (RSYN) have been cited as palliative treatments (Cruz-Esteban & Wilke, 1995; Gibbons et al., 2002). Synoviorthesis is used synonymously with RSYN; this term was originally adopted by French authors to reter to restoration of the synovium (Delbarre & Menkes, 1974). In the 1960s, radioactive gold-198 (^{198}Au) was applied to treat RA in human patients, but currently the most widely reported radiopharmaceutic for joint therapy is yttrium-90 (^{90}Y) colloid (Fellinger & Schmid, 1952; Virkkunen et al., 1967; Clunie & Ell, 1995). The procedure entails injection of a β emitting radiopharmaceutic directly into the joint to control and ablate inflammation (Ansell et al., 1963). Radiopharmaceutics, usually colloids or aggregates, are assumed to be quickly phagocytosed by synoviocytes and then distributed within the synovium, primarily at the surface (Ingrand, 1973). Diseases of the synovium can result in increased vascularization, edema, synovial layer proliferation, lymphocytic infiltration, and fibrosis (Johansson & Rejno, 1976). The objective of RSYN is to limit the inflammatory reaction. RSYN has been described as an effective local treatment for RA, hemophilic arthritis, and recurrent synovitis in human patients who have not responded to medical therapy (Jahangier et al., 2001; Siegel et al., 2001). Once the inflamed synovium is destroyed by direct radiation, the regenerated synovium is free of disease. Dosing of radiopharmaceutics is still based more on clinical experience than on experimental studies, and the exact dose required of a given radiopharmaceutic to achieve effective synovectomy remains unclear (Deutsch et al., 1993).

Synovial regeneration has been reported to occur within 6-9 weeks following RSYN in rabbits (Pavelka et al., 1975). The articular cartilage may sustain some degree of enzymatic degradation, which continues until synovial regeneration is complete (Mitchell & Cruess, 1967b). Traumatic injury of the synovial membrane can lead to chronic synovitis (Richardson, 2002) and contribute to the degenerative process by release of substances directly or indirectly harmful to the cartilage such as matrix metalloproteinases, prostaglandin E_2, free radicals, and cytokines (Palmer & Bertone, 1994). RSYN has been claimed to predispose the treated joint to radiation-induced chondrocyte injury and subsequent osteoarthritis (OA) (Pavelka et al., 1975; Meier-Ruge et al., 1976; Kerschbaumer et al., 1979). However, the risk of radiation-induced cartilage injury is generally considered small relative to the destruction induced by the evolution of the rheumatoid synovitis itself (Meier-Ruge et al., 1976).

Rabbits are the preferred animal for studying RSYN. Normal animals have been used to study leakage, dosimetry, and radiation-induced damage to articular cartilage (Deutsch et al., 1993). RSYN in humans is performed to treat recurrent synovitis to reduce the ongoing inflammation that promotes degradative changes in articular cartilage (Smith, 1999). The chronic arthritis produced by intra-articular injections of soluble protein antigens into

previously immunized rabbits (antigen-induced arthritis, AIA) provides an animal model that mimics RA (Paget & Gibofsky, 1979). Considerable differences do, however, exist between lapine AIA and human RA, and the effects of RSYN on AIA must be interpreted with caution (Deutsch et al., 1993; Hembry et al., 1993). In retired racehorses, natural degeneration of cartilage is expected because of their previous racing careers. The dorsal aspect of the fetlock joint in racehorses is especially vulnerable to injury during active training and racing (Pool, 1996). Thus, our study on horses provides a model for early naturally occurring degenerative joint disease.

Several new radionuclides and carriers have recently been studied for treatment of various joint disorders (O'Duffy et al., 1999; Shin et al., 2001; Ofluoglu et al., 2002; Lee et al., 2003a). Holmium-166 (^{166}Ho) is a radionuclide with excellent physical properties for synovectomy. A reasonably short half-life (1.1 days) ensures effective synovectomy without increased risk of unwanted radiation burden to normal organs. Betaradiation energy for radiosynovectomy (β_{max} 1.8 MeV) is suitable for depositing a substantial dose to the thickened synovium. The physical properties enable 90% of the radiation to penetrate to a depth of 2 mm in the thickened synovium (Johnson & Yanch, 1991). Ionizing γ radiation (81 keV) increases only slightly the total radiation to the body. A stable daughter is formed after radioactive decay. ^{166}Ho is bound to ferric hydroxide macroaggregate (FHMA) particles, which are small enough to be easily phagocytosed by the synovium but large enough to be retained in the joint (Hnatowich et al., 1978; Noble et al., 1983; Davis & Chinol, 1989).

RSYN has been shown to control clinical symptoms of RA in man during follow-up periods of longer than a year (Sledge et al., 1986; Zuckerman et al., 1987; Will et al., 1992). Moreover, a retrospective study of racehorses has claimed better racing results after arthroscopic synovectomy (Roneus et al., 1997), but only two experimental studies exist on the use of RSYN with samarium-153 hydroxyapatite microsphere (^{153}Sm M) in equine medicine (Yarbrough et al., 2000a, 2000b). Our experimental study assesses the effects of ^{166}Ho FHMA on the synovium and cartilage of normal rabbits and horses.

REVIEW OF THE LITERATURE

1. Anatomy and physiology of the synovial joint

1.1. Joint capsule

The joint capsule of a synovial or diarthrodial joint consists of a thick fibrous portion and a synovial membrane. The fibrous layer of the capsule comprises a dense tissue containing moderate amounts of collagen and elastic fibers. The fibrous layer contributes to the mechanical stability of the joint. The synovial membrane contains two mesenchymal layers, the subintimal, and the intima. The subintima is adjacent to the fibrous layer and is composed of fibrous, areolar, or adipose tissue depending on the function of and location within the joints. The intima is composed of a 1- to 3- cell-thick synoviocyte layer with no basement membrane. Synoviocytes consist of cells that have both secretory and phagocytic functions. Synoviocytes that phagocytose and pinocytose are type A or macrophage-like cells. Type B or fibroblast-like cells secrete hyaluronan and lubricin into the synovial fluid (Henderson, 1988). These molecules provide high viscosity to the synovial fluid (Poole et al., 2001). Synovial lining cells have been suggested to be regarded not as different cell entities but rather as different functional stages of cells (Henderson, 1988).

1.2. Synovial fluid

In healthy joints, a small volume of synovial fluid - a colorless or pale-yellow viscous liquid - occupies the intra-articular space. Synovial fluid is an ultrafiltrate of plasma. Most elements, except hyaluronan and lubricin, are derived from the blood. Synovial fluid contains mostly mononuclear cells (synoviocytes, monocytes, and lymphocytes), and the remainder of the cells are polymorphonuclear leukocytes. Normal equine synovial fluid contains <500 nucleated cells/μl (Todhunter, 1996). Chondrocytes in mature animals obtain their nutrients by diffusion from the synovial fluid (Hodge & McKibbin, 1969; Maroudas, 1970).

1.3. Articular cartilage

The articular cartilage is a hyaline cartilage. In the synovial joint, the hyaline cartilage forms a frictionless articulating surface that allows movement and transfer of loads between bones. Articular cartilage is mainly composed of an avascular and aneural extracellular matrix. The extracellular matrix contains water, collagen, proteoglycans, and other noncollagenous proteins.

Cartilage is divided into superficial, intermediate, and deep zones. The zones can be distinguished from one another by the varying arrangement of their cells and differences

in their matrix. In the superficial zone, the chondrocytes are flattened and parallel to the cartilage surface. Thin collagen fibrils are arranged parallel to the articular surfaces. In this zone, the large proteoglycan aggrecan is present in its lowest concentration (Poole et al., 2001). The superficial collagen fibers provides the highest tensile properties found in articular cartilage (Akizuki et al., 1986). The intermediate zone consists of rounded cells surrounded by an extensive extracellular matrix rich in proteoglycan aggrecan. Collagen fibrils are arcading, like branches on a tree. In the deep zone, the cell volume is at its lowest, and cells are often grouped in clusters. The collagen fibrils are larger and predominantly perpendicular to the surface. Aggrecan content is at a maximum in this deep zone. Adjacent to the zone and most remote from the articular surface, is the calcified zone. This calcified zone is separated microscopically from the uncalcified cartilage by a distinct line, the tidemark.

1.3.1 Chondrocytes

Chondrocytes are situated amongst an extensive extracellular matrix and have a cell volume that averages only 2% of the total tissue volume in articular cartilage of human adults (Stockwell & Meachim, 1979). All chondrocytes are surrounded by a narrow pericellular region that contains proteoglycans and minor collagens. This organization of the chondrocyte and its pericellular matrix is known as a chondron. The chondrons are surrounded by a territorial region that is present throughout the cartilage (Poole et al., 2001). The territorial regions are scattered in the interterritorial matrix, where the proteoglycan concentration is lower than in the territorial matrix. The turnover of matrix is regulated by chondrocytes under the control and influence of growth factors, cytokines, and mechanical stimuli (Poole, 1993).

1.3.2. Collagens

Type II collagen (90%) predominates in the articular cartilage. About half of the dry weight of the articular matrix is made of type II collagen, except in the superficial zone, where it accounts for most of the dry weight. The biosynthesis of type II collagen begins with activation of the *Col2α1* gene. During intracellular synthesis three identical α_1(II) chains wind round each other to form a triple helix. This procollagen molecule is stabilized by interchain hydrogen bonds between the α_1(II) chains. Extracellular conversion of a procollagen to a tropocollagen molecule necessitates the cleavage of amino and carboxy terminal propeptides, which border the procollagen molecule. In the amino and carboxy telopeptide domains, covalent crosslinks form between lysine residues in adjacent chains, making type II collagen fibrils very resistant to degradation (Poole et al., 2001). During fibril formation the tropocollagen molecules are arranged in a quarter-staggered array. The collagen framework is built before maturation; and adult articular cartilage has a limited capacity for repair.

Several minor collagens, especially types IX and XI, account for 10% of the total collagen content (Mendler et al., 1989). Minor collagen type IX is located on the surface of type II collagen fibrils, whereas type XI collagen is present both in the center of collagen fibrils and on fibril surfaces (Mendler et al., 1989). Both type IX and type XI increase the mechanical stability of the type II fibrillar network (Kuivaniemi et al., 1997; Blaschke et al., 2000).

1.3.3. Proteoglycans

Large proteoglycan aggregates are trapped in the collagen fibril network, and the integrity of this network is essential in retaining aggrecan molecules in the cartilage. The proteoglycan content accounts for about 25% of the dry weight of the cartilage matrix. The proteoglycans of articular cartilage fall into two broad categories: large aggregating proteoglycans and small proteoglycans. The rest are aggrecan degradation products which accumulate with age. The main proteoglycan, aggrecan, makes up 50-85% of the total proteoglycan mass. Aggrecan content is relatively poor in the superficial zone. Its concentration increases with increasing depth (Bayliss, 1986). The aggrecan core protein, a polypeptide that is about 2000 amino acids long, contains three globular domains. Two globular domains (G1 and G2) are at the hyaluronan-associated end. Aggrecan binds through its amino terminal G1 globular domain to hyaluronan (Poole et al., 2001). This binding is stabilized by the link protein. The majority of sulfated glycosaminoglycans (GAGs) are contained within an extended region situated between the G2 and G3 domains. A single aggrecan molecule includes approximately 100 chondroitin sulfate and 30-50 keratan sulfate side chains, and also 60-70 O- and N-linked oligosaccharides. Aggrecan shows an age-related decrease in size and enrichment in keratan sulfate relative to chondroitin sulfate. The carboxyterminal G3 domain might be of importance in binding to other molecules and regulating intracellular trafficking and processing of aggrecan (Halberg et al., 1988; Zheng et al., 1998). Biosynthesis of aggrecan starts in the rough endoplasmic reticulum with translation of gene transcripts for aggrecan core protein, followed by N-linked glycosylation and addition of keratan sulfate and chondroitin sulfate chains in the Golgi apparatus. After the GAG chains have been added, the proteoglycan molecules are transported to the plasma membrane and released into the pericellular matrix.

Hyaluronan, a nonsulfated GAG consisting of repeated N-acetylglucosamine and β-glucuronic acid molecules, is built as a free chain on the plasma membrane. Binding of several proteoglycan monomers to a single hyaluronan molecule results in a large aggregate that is trapped in the collagen fibril network. The binding of aggrecan monomers on hyaluronan occurs extracellularly. Water is bound to aggrecans, forming a gel-like structure in which water comprises up to 65-80% of the matrix volume. Glycosaminoglycans are highly negatively charged carbohydrate chains, and aggrecans can bind up to 50 times their weight in water. Cartilage has a tendency to swell, which is resisted by the collagen network. The presence of water in association with the hydrophilic and negatively

charged GAGs contributes to the compressive resilience of the articular cartilage (Kempson, 1975).

The small proteoglycan molecules only constitute 1-4% of the total mass of proteoglycans in articular cartilage, although they maybe as numerous as the large aggregating proteoglycans. Articular cartilage has been shown to contain at least four small proteoglycans, all of which are members of a small leucine-rich proteoglycan (SLRP) gene family (Iozzo, 1997). The SLRPs recognized in articular cartilage are biglycan, decorin, fibromodulin, and lumican. An important function of SLRPs seems to be the regulation of collagen fibrillogenesis (Vogel et al., 1984; Hedbom & Heinegård, 1993; Scott, 1996; Svensson et al., 1999; Reed & Iozzo, 2002).

2. Synovitis and arthritis

2.1. Synovitis

Virtually every racehorse has one or more episodes of primary capsulitis and synovitis during their racing career (McIlwraith, 1987). Characteristic clinical findings include heat, effusion, and pain with flexion of joints (Richardson, 2002). Primary synovitis in horses is characterized by inflammation of the synovium without gross disturbance of articular cartilage or disruption of major supporting structures (Todhunter & Lust, 1990). Primary synovitis is especially common in the metacarpophalangeal joint (Richardson, 2002). Ligamentous instability, trauma, or degeneration of the articular cartilage can lead to secondary synovitis (Todhunter & Lust, 1990). Microscopic changes in inflamed equine joints include increased vascularization, edema, infiltration of mononuclear cells, and hypertrophy and hyperplasia of the synoviocytes (Johansson & Rejno, 1976).

An inflamed synovial membrane has the ability to induce damage to the articular cartilage (Fell & Jubb, 1977). The main destructive mediators are interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Goldring, 2000; Bertone et al., 2001). IL-1 and TNF-α are produced by synoviocytes, mononuclear cells, and chondrocytes (Goldring, 2000). Cellular production of IL-1 and TNF-α requires an appropriate stimulus by bacteria, immune complexes, aggregated antibodies, matrix components, or lymphokines (Paget & Gibofsky, 1979; Sumi et al., 1986; Dinarello, 1988; Hardy et al., 1998; Homandberg, 1999; Kagari et al., 2003). The action of IL-1 alone or together with TNF-α on articular cartilage is multifaceted, with many different gene products being influenced either by stimulation or by suppression (van den Berg, 1999; Bertone et al., 2001). Catabolic cytokines can significantly upregulate matrix metalloproteinase (MMP) gene expression (Richardson & Dodge, 2000; Fernandes et al., 2002). These MMPs are the major enzymes involved in early structural changes in cartilage (Pelletier et al., 2001). The expression of inducible nitric oxide synthase, cyclooxygenase-2 (Cox-2), and phospholipase A2 is increased by OA chondrocytes when stimulated by IL-1 alone or in combination with TNF-α (Goldring, 2000). Products of these enzymatic activities, particularly nitric oxide and E-series

prostaglandins, have been associated with cartilage injury (Blanco et al., 1995; Hashimoto et al., 1998; Bertone et al., 2001; Kim et al., 2003).

Increased IL-1 levels are present in synovial fluid from osteoarthritic and inflamed equine joints (Morris et al., 1990; Alwan et al., 1991b; Theoret et al., 1998; Bertone et al., 2001). In humans, the level of TNF-α was found to be higher in synovial fluid obtained from rheumatoid joints than from osteoarthritic joints (Hopkins & Meager, 1988). Cytokines can reduce matrix synthesis at much lower concentrations than those needed to stimulate degradation (Poole, 1993). IL-1 inhibits both collagen and proteoglycan synthesis in young cartilage, while old cartilage is significantly less responsive (Nietfeld et al., 1990; MacDonald et al., 1992; Richardson & Dodge, 1997). IL-1 and TNF-α can also induce concomitant production of IL-6 and chemokine IL-8 (Pelletier et al., 1993; Goldring, 2000). Synovial fluid IL-6 concentration has been recognized as an excellent indicator of naturally acquired disorder in the equine joint (Bertone et al., 2001). Proinflammatory IL-8 plays an important role in acute inflammatory reaction; it attracts neutrophils and generates reactive oxygen metabolites (Djeu et al., 1990, Fernandes et al., 2002).

2.2. Enzymatic degradation of the extracellular matrix

The functional capability of articular cartilage rests with maintaining the content and structure of the principal extracellular molecules, collagen, and proteoglycan. In OA, both aggrecan and type II collagen are degraded by proteolytic attack induced by degradative cytokine IL-1 (Smith, 1999; Mort & Billington, 2001). The major proteases involved in early cartilage degradation are MMPs and aggrecanases. Other enzymes, such as stromelysins, gelatinases, and catephins, are also involved, but primarily as activators of MMPs (Pelletier et al., 2001).

Collagen fibrils are very resistant to most proteinases and only a few proteinases, known as collagenases, are capable of cleaving the triple helix. Collagenases belong to a group of MMPs which initiate the breakdown of native collagens by denaturating or unwinding the triple helix (Shingleton et al., 1996; Shlopov et al., 1997). They are a group of zinc-dependent endopeptidases involved in the physiological turnover of the extracellular matrix at neutral pH (Birkedal-Hansen et al., 1993). They are secreted as latent proenzymes and activated extracellularly (Goldring, 2000). Rodent interstitial collagenase (MMP-13), neutrophil collagenase (MMP-8), interstitial collagenase (MMP-1), and membrane type 1 MMP (MMP-14) are known to cleave the triple helix of the collagen molecule (Shingleton et al., 1996; Goldring, 2000). Evidence suggests that MMP-13 is the most important proteinase responsible for the resorption cartilage in osteoarthritis (Poole et al., 2003b).

The aggrecanases (adamalysin subfamily of metalloproteinases; ADAMTS proteinases) play a pivotal role in chondrocyte-mediated aggrecan cleavage. Currently three aggrecanases, ADAMTS-1, ADAMTS-4, and ADAMTS-5, are known to cleave aggrecan

during the development of arthritis (Mort & Billington, 2001; Nagase & Kashiwagi, 2003). Recently, the role of IL-1 in mediating degradation of aggrecan by the aggrecanases has been studied extensively (Arner, 2002; Nagase & Kashiwagi, 2003).

Collagenases are not the only metalloproteinases associated with early cartilage structural changes. Stromelysin-1 (MMP-3) can cleave aggrecan molecules and is an important activator of latent collagenases (Murphy et al., 1987; Milner et al., 2001). An increase in gelatinase A (MMP-2), gelatinase B (MMP-9), and matrilysin (MMP-7) expression has been shown in osteoarthritic cartilage (Imai et al., 1997; Tsuchiya et al., 1997; Ohta et al., 1998). Cathepsins B, D, and L contribute to further degradation of various matrix components (Mort & Billington, 2001).

A complex array of regulatory mechanisms and endogenous inhibitors is normally sufficient to restrict the activity of any extracellular proteinase (Dean et al., 1989; Martel-Pelletier et al., 1994). Amongst these are tissue inhibitors of metalloproteinases which bind to the active site of the MMP and inhibit its catalytic activity. In OA and RA, evidence indicates an imbalance between MMPs and their natural inhibitors that contributes to the cartilage degradation (Martel-Pelletier et al., 1994; Reynolds, 1996).

2.3. Osteoarthritis

In humans, osteoarthritis is defined as noninflammatory arthropathy (Hedbom & Hauselmann, 2002). The pathogenesis of OA includes mechanical, biochemical, and genetic factors that contribute to the imbalance between cartilage matrix degradation and synthesis (Goldring, 2000). Independent of the initiating factor, enzymatic degradation is now recognized as a central feature of OA (Ishiguro et al., 2002; Caron, 2003). Chondrocytes and synoviocytes are the main sources of enzymes responsible for cartilage breakdown (Hollander et al., 1995; Vankemmelbeke et al., 1998).

A functional collagen fibrillar network is essential for healthy articular cartilage since there is no indication of extensive cartilage repair once the collagen fibrils are broken down (Poole et al., 2001). In early OA, type II collagen damage is restricted to pericellular and superficial sites (Wu et al., 2002). Superficial collagen damage leads to fibrillation (Hollander et al., 1994) and increased water content of the cartilage surface (Brocklehurst et al., 1984). Clonal formation of chondrocytes is often observed (Ishiguro et al., 2002). In more advanced OA, increased bone formation is characterized by periarticular osteophyte formation and sclerosis of subchondral bone. Cleavage of the collagen network results in progressive loss of cartilage, causing deep fibrillation and vertical clefts. Calcification leads to formation of new tidemarks and, finally, degenerative changes result in a complete loss of articular cartilage, exposing the underlying subchondral bone (Buckwalter & Martin, 1995).

Although degradation of the collagen network is an essential prerequisite for OA, enhanced loss of proteoglycans is also closely associated with destruction of articular

cartilage (Ishiguro et al., 2002). The sites of early type II collagen damage and loss of proteoglycan coincide (Hollander et al., 1995). The loss of proteoglycans imposes an increasing load on collagen fibrils in the softer articular cartilage and induces collagenase accessibility to these fibrils (Pratta et al., 2003). In early OA, a decrease in hyaluronan content, diminution of proteoglycan aggregates and monomers, and decreased aggregation of the monomers have been reported (McDevitt & Muir, 1976; Sweet et al., 1977; Vasan, 1980; Tyler, 1985; Martel-Pelletier et al., 1988; Rizkalla et al., 1991).

The levels of mRNA for cartilage matrix components, especially type II collagen, are high in growing and immature cartilage, but adult cartilage has a very limited capacity for matrix synthesis (Poole, 2003a). However, in early OA, there are distinct attempts at cartilage repair in adult cartilage (Eyre et al., 1980; Sandy et al., 1984). The transcriptional factor Sox9 is required for transcriptional upregulation of Col2α1 gene (Lefebvre et al., 1997). Elevated synthesis of type II collagen and proteoglycans is associated with attempted cartilage repair during early OA in humans (Poole, 1993; Aigner et al., 1999). Elevated expression of type II collagen, but not aggrecan core protein mRNA, has been shown in OA in horses (Fehr et al., 2000).

2.4. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease accompanied by signs of systemic disturbance. The autoimmune phenomena in humans are probably genetically determined (Winchester et al., 1992). To establish a diagnosis of RA, the production of "rheumatoid factors" should be established (Harris, 1990). However, rheumatoid factors can also be found in other conditions such as viral and liver diseases (Grassi et al., 1998b). Rheumatoid factors are antibodies which react with altered host immunoglobulins to form immunocomplexes (Paget & Gibofsky, 1979). The resultant immune complexes are deposited on the synovium and articular cartilage, initiating inflammatory and degradative processes (Paget & Gibofsky, 1979; Sumi et al., 1986). In rheumatoid patients, subacute or chronic nonsuppurative joint inflammation usually affects three or more joints (Harris, 1990). Morning stiffness in and around the joints is a sign typical of RA (Grassi et al., 1998a). Evidence suggests that transcription factor NF-κB plays a critical role in the activation and proliferation of synoviocytes (Yamanishi & Firestein, 2001). Histological changes include hyperplasia of synoviocytes and an inflammatory cellular infiltrate composed mainly of mononuclear cells. Numerous villi and swollen masses of redundant synovial tissue fill the joint. Inflammation often leads to formation of pannus, a fibrovascular granulation tissue arising from the perichondral synovial membrane (Kobayashi & Ziff, 1975). The pannus is firmly attached to the articular cartilage, and degradation by proteolytic enzymes from monocytes and fibroblasts eventually leads to destruction of the articular cartilage (Harris et al., 1977). Articular cartilage degeneration also occurs in the deep layers near the bone-cartilage interface (Dodge & Poole, 1989). Subchondral bone inflammation leads to erosive bone changes characteristic of RA (Harris, 1990). In animals, RA has been described in dogs, but there are no reported cases in horses (Coughlan et al., 1998; Bertone, 2002).

3. Treatment of synovitis in horses

The basic treatment of primary synovitis entails rest, limited motion, and physical therapy. A full racing schedule often limits this option in fit horses. Intra-articular therapy is an alternative, common treatment for synovitis. Certainly the most widely used intra-articular therapy for exercise-associated articular pain is corticosteroids. Being the most potent anti-inflammatory agents available, they are considered beneficial in treating the synovitis-capsulitis entity of traumatic arthritis in the horse (Trotter, 1996). The blockage of phospholipase A_2 leads to reduced formation of eicosanoid derivatives (Higgins & Lees, 1984). Corticosteroids eliminate pain, decrease joint swelling, and stabilize cell membranes, thus preventing recruitment of inflammatory mediators, destructive enzymes, and inflammatory cells into the joint (Trotter, 1996). However, their use, particularly repeated use, remains controversial in equine athletes due to their inhibitory effects on chondrocyte metabolism (Dechant et al., 2003).

Medical application of hyaluronan, namely sodium hyaluronate (NaHA), is successfully used for treatment of OA in humans and horses. Suppression of PGE_2 production by the synoviocytes, decreased vascular permeability, and increased synovial fluid hyaluronan content are offered as possible mechanisms for the therapeutic effects of NaHA (Asari et al., 1998). Symptomatic improvement, i.e. reduced pain and improved joint mobility, can be dramatic (Gotoh et al., 1993). In vitro, NaHA stimulates proteoglycan synthesis by equine chondrocytes (Frean et al., 1999). Cartilage-sparing effects by high molecular weight NaHA were reported after partial menisectomy in rabbits (Kikuchi et al., 1996). In the horse, NaHA is most effective in treating acute synovitis-capsulitis rather than more advanced disease (Caron & Genovese, 2003). Systemic administration of NaHA was effective in improving clinical signs associated with joint disease caused by osteochondral fragmentation (Kawcak et al., 1997).

Suggested mechanisms for the favorable effects of polysulfated glycosaminoglycans (PSGAGs) include chondroprotection and inhibition of PGE_2 synthesis (Altman et al., 1989; Frean & Lees, 2000). PSGAGs are principally made up of chondroitin sulfate. Cartilage-sparing effects are attributed to the inhibition of degradative enzymes (Altman et al., 1989). There is in vitro proof that aggrecanase action is inhibited by another chondroprotective drug, pentosan polysulfate (Ghosh, 1999). Clinical experience suggests that pentosan polysulfate is effective in reducing lameness in racehorses with OA (Little & Ghosh, 1996).

Systemic administration of nonsteroidal anti-inflammatory drugs (NSAIDs) has been used to stop inflammatory processes and enable increased weight-bearing. Their main effect is inhibition of the prostaglandin pathway of arachidonic acid metabolism via the blockage of induced cyclo-oxygenase (Vane, 1976). However, the effects of COX inhibitors on cartilage structure are unknown (Pelletier et al., 2001). Most NSAIDs are ineffective in inhibiting IL-1-induced cartilage degradation (Rainsford, 1985). In addition, they may have inhibitory effects on chondrocyte metabolism (Fujii et al., 1989).

4. Surgical synovectomy

With the development of instrumentation and surgical techniques, arthroscopy has surpassed arthrotomy in diagnosis and management of joint diseases. Advanced arthroscopic techniques have enabled removal of 95% of diseased synovial tissue in the human rheumatoid knee joint, providing long-term relief of pain and swelling as well as improvement in joint function (Rosenberg et al., 1996). However, arthroscopic synovectomy does not halt progression of RA (Gibbons et al., 2002; Roch-Bras et al., 2002). Magnetic resonance imaging (MRI) signs of recurrent synovitis may be present within two months of surgical synovectomy (Ostergaard et al., 2001).

Indications and potential benefits of surgical synovectomy in horses have not been properly determined (Caron & Genovese, 2003). A retrospective study of racehorses claimed better racing results after surgical synovectomy (Roneus et al., 1997). However, the physiologic significance of the slow regenerative capacity of equine synovium is not known. Avillous appearance of the synovium and poor regeneration of synovial lining were reported three months after surgical synovectomy in horses (Theoret et al., 1996; Doyle-Jones et al., 2002).

5. Chemical synovectomy

Chemical synovectomy in humans has been attempted with the aid of various products such as osmic acid, the alkylating agents nitrogen mustard and thio-tepa, methotrexate, and antibiotics (Cruz-Esteban & Wilke, 1995). The risk of chondrocyte necrosis and systemic toxicity has decreased the popularity of chemical synovectomy (Niculescu et al., 1976; Molho et al., 1999). However, osmic acid and rifampicin are still occasionally used to treat chronic hemophilic synovitis (Molho et al., 1999; Rodriguez-Merchan & Wiedel, 2001).

6. Radiation synovectomy

6.1. Radionuclides

Radiation synovectomy has been used in human medicine for over 50 years to treat RA (Fellinger & Schmid, 1952). Many radionuclides have been employed for synovectomy, including holmium-166 (^{166}Ho, β_{max} 1.8 MeV), dysprosium-165 (^{165}Dy, β_{max} 1.3 MeV), yttrium-90 (^{90}Y, β_{max} 2.2 MeV), gold-198 (^{198}Au, β_{max} 1 MeV), phosphorus-32 (^{32}P, β_{max} 1.7 MeV), rhenium-186 (^{186}Re, β_{max} 1.1 MeV), and erbium-169 (^{169}Er, β_{max} 0.3 MeV). They all emit primarily β radiation; and ^{198}Au, ^{166}Ho, ^{165}Dy, and ^{186}Re also emit γ radiation. The radionuclides most frequently used are ^{90}Y and ^{198}Au (Zuckerman et al., 1987). However, ^{198}Au is being abandoned in favor of the pure β emitter ^{90}Y (Clunie & Ell, 1995). The ideal radionuclide is a β emitter with low γ emissions and a maximum tissue

penetrance of less than 10 mm. It should have sufficient MeV for effective synovectomy and short half-life to minimize extra-articular leakage (Johnson & Yanch, 1991). Furthermore, it should be chemically pure, nontoxic, and economically viable (Zuckerman et al., 1987). The use of different radionuclides varies depending on the size of the joint and the energy of the radionuclide. ^{90}Y is suitable for the knee and joints with a greatly thickened synovium because of its deep tissue penetration (mean = 3.8 mm, max = 11 mm) (Johnson & Yanch, 1991). For intermediate-sized joints, ^{186}Re has been recommended, and for the smallest joints ^{169}Er is best suited (Clunie & Fischer, 2003). Short physical half-lives are desirable since leakage of radioactivity from the joint takes time, and nuclei that decay before leaking do not subject the patient to unnecessary extra-articular radiation (Johnson et al., 1995). The half-life of ^{165}D is very short (0.1 day) which makes it difficult to use for clinical purposes due to the requirement of fast transportation times from manufacturer to patient. From a clinical viewpoint, ^{166}Ho is much more practical for widespread use because of its longer half-life (1.1 days) (Hosain et al., 1990).

6.2. Carriers for radionuclides

If an unconjugated radionuclide were to be injected directly into a joint, it would rapidly diffuse out due to its small molecular size. This would increase radiation to normal organs. Therefore, radionuclides must be attached to nondiffusible particles. Properties of an ideal particle include biodegradability, good synovial uptake, stable preparation in vitro, little or no leakage from the joint, quick, easy, and reproducible preparation, and no toxic or allergenic properties (Sledge et al., 1977). Typically, radionuclides are attached to colloids or larger aggregates (Srivastava & Dadachova, 2001).

The disadvantage of using inorganic colloid particles is significant extra-articular leakage (Sledge et al., 1977; Noble et al., 1983; Johnson & Yanch, 1991). The leakage can be reduced if the patient stays in bed for few days or the leg is splinted after injection (Oka et al., 1971; Clunie & Fischer, 2003). Of particular concern is transport of particles through lymphatics to regional lymph nodes, which causes a high radiation dose to circulating lymphocytes. The small particle size of ^{198}Au results in high extra-articular leakage and chromosome damage, and hence, radiation risk to normal organs (Virkkunen et al., 1967). A significantly elevated number of chromosome anomalies was also observed after treatment with ^{90}Y (de la Chapelle et al., 1972). Lymphocyte damage might increase the risk for a carcinogenic effect, i.e. development of leukemia (Stevenson et al., 1973). However, a long-term study found no evidence of increased risk for cancer after ^{90}Y synovectomy of the knee joint (Vuorela et al., 2003). Beta emitters combined with tin-colloids can prevent leakage by forming particles of appropriate size (Lee et al., 2003a).

The problem of leakage of radioactivity from the joint can be diminished by using larger aggregates (Noble et al., 1983). Particles of 1-20 μm are ideally suited for RSYN; they are small enough to be phagocytosed by synoviocytes but large enough to be retained in the joint (Hnatowich et al., 1978; Noble et al., 1983). The radionuclide ^{166}Ho is coupled with

ferric hydroxide macroaggregate particles (FHMA, size 3-8 μm), which are a hundred times larger than traditional colloids (Ingrand, 1973; Penttilä et al., 1998). The affinity of the synovium for FHMA is excellent since iron in FHMA is taken up well by the synoviocytes (Ball et al., 1964).

6.3. Dose absorbed by the synovium

Precise calculations of the dose absorbed by the inflamed synovium are difficult for many reasons. Estimation of the thickness of the synovial membrane and villous formation are, for instance, problematic. Some authors have attempted to calculate the absorbed dose by analyzing simple schemes of distribution (Ingrand, 1973). However, the estimated dose absorbed by the synovium was consistently inaccurate as a result of the simplified assumptions utilized in calculations (Johnson & Yanch, 1991).

Most published studies employ absorbed dose profiles from a mathematical model using joint phantoms to determine an appropriate radionuclide and to estimate the proper dose (Deutsch et al., 1993). Mathematical calculations of β particle dosimetry assume uniform intra-articular distribution of radiopharmaceuticals (Johnson & Yanch, 1991). These profiles reveal the dose absorbed by major components of the arthritic synovial joint as a function of penetration distance (Johnson & Yanch, 1991). The profiles can be used to select the proper radionuclide for individual joints and specific synovium conditions. If the volume of synovial tissue is known, advanced absorbed dose factors versus depth profiles help to evaluate dose rates even more accurately (Johnson et al., 1995).

6.4. Experimental studies of intra-articular effects

In horses, RSYN with samarium-153 hydroxyapatite microspheres (^{153}Sm M) has resulted in painful synovitis (Yarbrough et al., 2000a). Overt lameness improved within a few days, but effusion and edema persisted for two weeks. Joints studied 14 days after RSYN showed focal necrosis of the synovial intima. The pattern of synovial destruction was similar between low- and high-dose joints, but joints injected with the high dose showed more complete removal of the synovial lining (Yarbrough et al., 2000a). While treatment with ^{153}Sm M could not suppress synovial effusion associated with joint disease caused by osteochondral fragments, ^{153}Sm M itself did not harm the articular cartilage (Yarbrough et al., 2000a, 2000b).

Several studies have shown good and uniform uptake of ^{90}Y colloid by the synovium (Webb et al., 1969; Isomäki et al., 1972; Ingrand, 1973). In histological examination, fibrinoid necrosis, regression of synovial villi, reduction of cellular infiltrations, extensive fibrosis, and occlusion and sclerosis of smaller synovial vessels and capillaries have been observed after ^{90}Y treatment (Pavelka et al., 1975; Meier-Ruge et al., 1976; Myers et al., 1989). The severity of synovial abnormalities has varied between different individuals and

within individual joints (Pavelka et al., 1975; Sledge et al., 1987; Myers et al., 1989). In rabbits, full regeneration of the synovial membrane has occurred within 6-9 weeks following RSYN (Mitchell & Cruess, 1967a; Pavelka et al., 1975; Gomar & Arquelles, 1978).

One of the concerns regarding RSYN is its potential to cause cartilage damage. In addition to direct radiation damage, synovectomy has been suggested to result in some degree of enzymatic degradation of cartilage, which continues until synovial regeneration is complete (Mitchell & Cruess, 1967b). Mitotic activity of chondrocytes (Anderson, 1977; Stockwell & Meachim, 1979) and a higher rate of cartilage matrix synthesis in immature animals compared with adult animals (Salminen et al., 2001) might make young articular cartilage susceptible to irradiation. Suggested radiation-induced injuries include chondrocyte necrosis, swollen chondrocytes, and pycnotic, hypertrophic, and irregular grouping of cartilage cells (Meier-Ruge et al., 1976; Ubios et al., 1978; Kerschbaumer et al., 1979).

An antigen-induced arthritis (AIA) model in rabbits is typically exploited when screening potential synovectomy agents for treatment of RA (Deutsch et al., 1993). When progression of AIA was followed, beneficial effects of ^{90}Y treatment outweighed the harmful effects of radiation (Meier-Ruge et al., 1976). The beneficial effects correlated with reduction of the inflamed synovium and preservation of articular cartilage (Meier-Ruge et al., 1976).

6.5. Clinical use in man

6.5.1. Rheumatoid arthritis

The primary treatment for RA consists of medical treatments aimed at controlling synovial inflammation. Usually RA can be satisfactorily controlled with disease-modifying antirheumatic drugs, corticosteroids, or NSAIDs (Goldbach-Mansky & Lipsky, 2003; Mullan & Bresnihan, 2003). The criteria for RSYN include persistent synovitis which has not responded to multiple intra-articular injections of long-acting corticosteroids (Jones, 1993; Rosenberg et al., 1996; Clunie & Fischer, 2003). Therapeutic interventions need to be implemented before the joints have undergone severe rheumatic damage (Harris, 1990). Theoretically, progression of joint damage might be inhibited by RSYN (Gobel et al., 1997); however, there is little or no proof that radiopharmaceuticals do more than alleviate symptoms (Deutsch et al., 1993).

Assessment of therapeutic effects in clinical trials has been based upon improvements in clinical parameters based primarily upon the physical examination. These parameters include range of joint motion, presence and size of joint effusion, crepitus with motion, circumference of the knee at midpatella, local pain, and patient satisfaction (Deutsch et al., 1993). The problem in assessing clinical response lies in the fluctuating history of rheumatoid joint effusions and difficulties in quantifying clinical signs.

Scintigraphy and color ultrasound examination of the rheumatoid synovium are useful imaging procedures for objective assessment of synovial inflammation (Pons et al., 1996; Terslev et al., 2003). MRI of the knee is the preferred technique to assess thickness of the synovium (Alonso-Ruiz et al., 1998; Pirich et al., 1999; Lee et al., 2003b). MRI studies suggest effective long-term (>1 year) suppression of synovial lining hyperplasia after RSYN (Alonso-Ruiz et al., 1998; Pirich et al., 1999; Lee et al., 2003b).

Preliminary results of RSYN with ^{166}Ho FHMA suggest that 71% of patients with RA have partial or complete resolution of clinical signs after synovectomy (Ofluoglu et al., 2002). Clinical trials report a 60-70% success rate after ^{90}Y synovectomy (Doyle et al., 1977; Sledge et al., 1986), but the results vary depending on the joint involved (Jahangier et al., 2001). However, most RSYN studies have been poorly designed; the studies have not been blinded and have had insufficient sample sizes (Jones, 1993). A critical reanalysis based on randomized controlled trials indicates only a marginal effect of ^{90}Y (Jones, 1993; Heuft-Dorenbosch et al., 2000). In a double-blinded randomized trial, effects of ^{153}Sm M treatment with cortisone did not differ from cortisone treatment alone one year after treatment (O'Duffy et al., 1999). These disappointing findings might be associated with insufficient action of the radionuclide on the diseased synovium (Guaydier-Souquieres et al., 1989).

6.5.2. Other arthritides

Hemophilia is a sex-linked inherited disorder characterized by protracted hemorrhages in various sites including the joints. The underlying defect in the hemophilic patient is abnormally slow coagulation of the blood. Repeated episodes of bleeding lead to chronic synovitis, proliferation of the synovial lining, and formation of villous projections. Inflammation is seen by the presence of neutrophils, and later, by macrophages, lymphocytes, and plasma cells infiltrating the subintimal tissues. Obvious benefits have made RSYN a popular treatment for chronic hemarthrosis in hemophilic human patients (Alonso-Ruiz et al., 1998; Pirich et al., 1999; Rodriguez-Merchan & Wiedel, 2001).

Pigmented villous overgrowth and hemarthrosis are typical of pigmented villonodular synovitis in man. The etiology of villonodular synovitis is unknown, but the crucial change is massive growth of synovium inside the joint composed of lipid- and hemosiderin-laden cells in the subintimal tissue (Ghadially, 1983). Articular cartilage eventually suffers degeneration and joint destruction follows. In the initial trials, only 40% of clinical patients benefited from RSYN (Koptan et al., 1974). Better results were reported with combination therapy of surgical debulking and RSYN (Kat et al., 2000).

6.5.3. Side-effects

Reactive synovitis is a frequent sequela of RSYN, and whenever possible, simultaneous corticosteroid injection is recommended to decrease synovial flare (Lee et al., 2003a).

Inappropriate selection of a radionuclide has resulted in skin necrosis in finger joints (Gumpel, 1978). Flushing of the needle is advised to prevent needle tract necrosis (Menkes, 1979). Radiopharmaceutical allergy and septic arthritis are rare complications of RSYN (Clunie & Fischer, 2003).

AIMS OF THE STUDY

The aims of this study were as follows:

(i) to determine the effects of experimental ^{166}Ho FHMA treatment on the synovium of rabbits and horses (I, III),

(ii) to determine the distribution of radioactivity and risk of leakage after experimental ^{166}Ho FHMA injection (I, III),

(iii) to determine the effects of experimental ^{166}Ho FHMA treatment on the articular cartilage of rabbits and horses (II, IV).

MATERIALS AND METHODS

1. Holmium-166 ferric hydroxide macroaggregate

^{166}Ho is a product of the neutron activation of ^{165}Ho and is predominantly a β emitter (β_{max}1.8 MeV) with radiotherapeutic properties appropriate for therapy. It also emits γ radiation (81 keV, 6%) detectable at scintillation imaging. In these studies, we used ^{166}Ho FHMA, a sterile and isotonic combination of ^{166}Ho and macroaggregate FHMA. Analysis of particle size distribution showed that 75% of particles were between 3 μm and 20 μm in diameter, and no particles were smaller than 0.2 μm. ^{166}Ho FHMA was prepared by MAP Medical Technologies OY (Tikkakoski, Finland).

2. Animals and study protocol

2.1. Rabbits

Studies included 59 growing (n=29, mean age 3.7 months, range 106-122 days, mean weight 2.4 kg) and mature (n=30, mean age 1 year 3 months, range 446-519 days, mean weight 5 kg) New Zealand white rabbits. The rabbits were purchased from the National Laboratory Animal Center (Kuopio, Finland). During the studies rabbits were housed in individual stainless steel cages. Fifty-one rabbits were used for analysis of synovium, synovial fluid, and articular cartilage (Table 1). A volume of 0.4 ml of ^{166}Ho FHMA was injected into one knee. The contralateral control knee was injected with 0.4 ml of nonradioactive ^{165}Ho FHMA. ^{166}Ho FHMA injections were given using an anterior subpatellar approach. The mean dose of ^{166}Ho FHMA in mature rabbits was 121 MBq and in young rabbits 75 MBq (I, II). For the autoradiography study, eight mature rabbits were sacrificed 1-5 days following ^{166}Ho FHMA injections. ^{166}Ho FHMA was injected into one knee joint as described above. The mean dose was 71 MBq/joint (I).

Table 1. Number of rabbits studied after ^{166}Ho FHMA injections.

	Young rabbits (n=29; av. age 3.5 months)			Old rabbits (n=22; av. age 15 months)		
Group	4 days [a]	2 months	1 year	4 days	2 months	1 year
Treated	8	9	9	6	7	7
NC	1	1	1	1[b]	1[b]	1

NC, Not treated.

[a] Time-point that rabbits were studied after ^{166}Ho FHMA injections.

[b] These groups of old rabbits shared an agematched negative control animal.

2.2. Horses

Six adult mixed-breed horses (all mares, mean age 9 years, range 6-14 years, mean weight 475 kg, range 444-495 kg) were used. Horses had been raised and trained on private farms before being sold to the University of Helsinki. Horses had no signs of joint illness based on physical examination and had normal morphology based on radiographs of metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints. Radiation synovectomy of one of the fetlock joints was performed using ^{166}Ho FHMA, and the opposite control joint was injected with nonradioactive ^{165}Ho FHMA. Injections were performed under aseptic conditions through the lateral collateral sesamoidean ligament with the joint flexed. Injections into the MCP joints were performed 2 months and into the MTP joints 5 days before the animals were sacrificed (Figure 1). During the experiment the horses were kept in box stalls or paddocks. One control MTP joint was discarded from the study due to a wound infection after arthroscopy that required intra-articular NaCl flushing. The doses in horses were 688.2 MBq, 1006.4 MBq, 1154.4 MBq, 962 MBq, 1147 MBq, 962 MBq, 1050.8 MBq, 958.3 MBq, 999 MBq, 1069.3 MBq, 1043.4 MBq, and 958.3 MBq (mean 1000 MBq). Injected activity was determined by assaying the amount of activity in the syringe before and after injection in a well counter (Isaocal II, Vinten, UK), which was calibrated for ^{166}Ho. The dose was extrapolated from a preliminary human study (Ofluoglu et al., 2002). Using the dosimetric calculations, a target dose of 1000 MBq yields a dose of 212 Gy to the equine joint (Johnson & Yanch, 1991). The study protocols were approved by the Animal Care and Use Committee of the University of Helsinki.

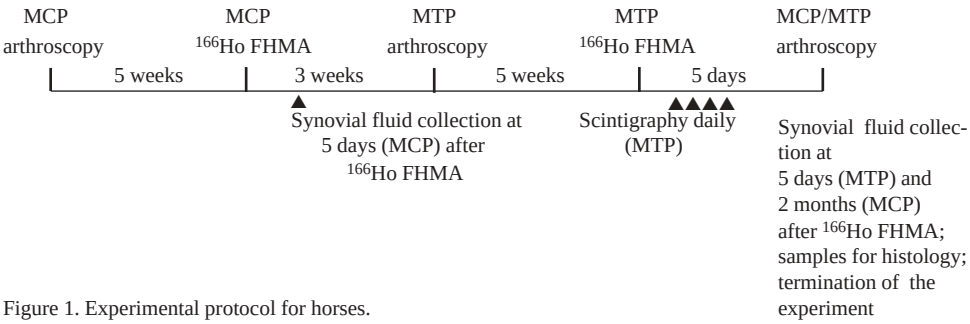

Figure 1. Experimental protocol for horses.

3. Clinical signs after RSYN (I, III)

Rabbits were inspected daily for any signs of discomfort. Horses were examined daily for signs of lameness at walk, and the limbs were inspected closely for signs of edema and joint effusion after ^{166}Ho FHMA treatment. Lameness at walk was evaluated daily for 5 days after ^{166}Ho FHMA treatment. Eight weeks after ^{166}Ho FHMA treatment, lameness at trot was evaluated and scored (Swanson, 1984).

4. Autoradiography of rabbits (I)

For the whole knee autoradiography study, 5 rabbits were sacrificed 24 hours apart (1-5 days) after ^{166}Ho FHMA. The dorsal joint capsules were studied 1, 2, and 3 days after ^{166}Ho FHMA injection. A detailed description of the autoradiography method is given in Study I.

5. Scintigraphy of horses (III)

The ^{166}Ho FHMA-treated MTP joints were examined daily for 5 days postinjection with scintigraphy. Static, straight lateral, 60-second images were acquired using a large-field-of-view gamma camera with 256 X 256 matrix size and a low-energy parallel-hole high-resolution collimator. Before image acquisition, the camera was calibrated for the γ radiation energy of ^{166}Ho (81 KeV) with a 20% window. Count rates were recorded and hand-drawn regions of interests were drawn around the proximal plantar joint pouch and the rest of the joint area to measure relative distribution of radioactivity in these regions.

6. Arthroscopy of horses (III, IV)

Arthroscopic examinations of horses were performed 5 weeks before the ^{166}Ho FHMA injections and were repeated immediately before the experiment was terminated (Figure 1). With arthroscopy, soft-tissue changes were evaluated (III) and stiffness data were obtained for the condylar surface and the sagittal ridge on the medial side of the dorsal joint area from treated and control joints (IV) (Figure 2A). Compressive testing of articular cartilage was performed with an arthroscopic indentation instrument validated for stiffness measurements of thin articular cartilage (Artscan 1000, Artscan Oy, Helsinki, Finland) (Lyyra-Laitinen et al., 1999). The contact plate was modified, i.e. elevated and reduced in size, to enable measurements of small cartilage areas using a pressing force of 7 N.

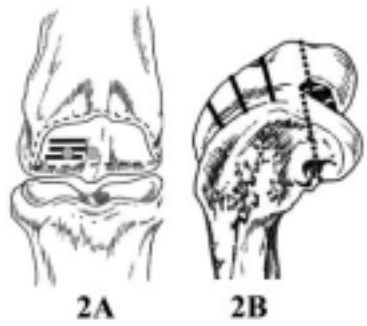

Figure 2. A schematic presentation of the sampling sites in equine and lapine joints. Three slices (black bars) of 2 mm in thickness were cut for morphometric and biochemical studies. (A) Two cartilage areas on the dorsal joint surface of horses (spots with stripes) were used for biomechanical analyses. Cartilage was harvested from the dorsal condylar surface (area inside the broken lines) for Northern blot. (B) The thick broken line indicates where the lapine condyles were cut for mRNA analysis of cartilage-specific genes

7. Histopathological study of the synovium (I, III)

Synovium samples were collected from the dorsal aspect of the knee joint of mature rabbits (4 days, 2 months, and 1 year after RSYN) and from the midline of the dorsal and proximal palmar and proximal plantar joint pouches of horses (5 days and 2 months after RSYN). For histological examination, samples were fixed in 4% formaldehyde in phosphate-buffered saline, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (HE). Ferric iron in the synovial tissue was demonstrated by Pearls' Prussian Blue staining and calcification using the von Kossa silver method.

8. Synovial fluid (I, III, IV)

Synovial fluid wash samples of mature rabbits were studied 4 days and 2 months after ^{166}Ho FHMA injections. Joint fluid samples were analyzed for total white blood cell count and total protein concentration. For total protein concentration, the samples were centrifuged at 5200*g* for 5 mintes, and the cell-free supernatants were collected. The total protein concentration was determined colorimetrically by the biuret method.

Synovial fluid samples from horses were collected 5 days and 2 months (only MCP joints) after RSYN (Figure 1). The synovial fluid was diluted 1:10 with 0.9% NaCl before analysis of total white blood cell count in a Bürker chamber. For total protein and GAG levels, samples were centrifuged at 8000*g* for 10 minutes, and the supernatants were collected. For analyses of GAGs, samples were digested with papain for 2-3 hours at 60^{o}C. The concentration of GAGs was measured in a 1.9-dimethylmethylene blue binding assay (Farndale et al., 1986). Analysis of residual radioactivity in synovial fluid was performed by liquid scintillation counting (Winspectral™, Wallac, Turku, Finland) using a water-soluble scintillation cocktail (Optiphase Hisafe III, Wallac, Turku, Finland). The results were compared with those of known low-activity ^{166}Ho FHMA standards.

9. Articular cartilage analyses (II, IV)

9.1. Metabolic labeling of articular cartilage

Three slices of articular cartilage with subchondral bone were cut from the medial condylar surface of the metatarsal III and metacarpal III bones of horses (Figure 2A) and from the distal femur of rabbits (Figure 2B). Cartilage samples from horses were collected 5 days and 2 months after RSYN, and from rabbits 4 days, 2 months, and 1 year after RSYN. One slice was taken for histology, quantitative autoradiography, and densitometric analyses of GAGs, another one for total sulfate incorporation and uronic acid analyses, and the third for electrophoretic analysis of proteoglycans. All slices were metabolically labeled with sulfur-35 (^{35}S) sulfate. After labeling, the slices were washed to separate most of the unincorporated ^{35}S sulfate precursor.

9.2. Cartilage histology and assessment of the superficial collagen network

Specimens for histology were fixed in 4% formaldehyde at 4°C for 20 hours, followed by decalcification in buffered 10% EDTA and 4% formaldehyde for 12 days at room temperature, dehydration with alcohol, and embedding in Paraplast Plus wax. Specimens were cut into 3-μm-thick sections for safranin-O staining, and 5-μm-thick sections for HE staining and subsequent histologic assessment. In rabbits, quantitative polarized light microscopic analyses were performed to assess the superficial collagen network (II). In horses, degenerative changes were evaluated from the HE-stained sections according to the following scale: 0 = normal cartilage, 1 = fibrillation, 2 = horizontal fibrillation, 3 = vertical fibrillation, and 4 = erosion of cartilage (IV).

9.3. Digital densitometry of glycosaminoglycans and thickness measurement of uncalcified cartilage

Densitometric determination of GAG concentration in safranin-O-stained sections was carried out as described previously (Kiviranta et al., 1985; Panula et al., 1998; Suominen et al., 2001). The measured area spanned from the cartilage surface to the tidemark. The thickness of the uncalcified articular cartilage was measured and divided into 12 fractions, each corresponding to 1/12 of the total thickness of the cartilage and with a constant width of 620 μm. The two most superficial fractions represented the superficial, the next five fractions the intermediate, and the deepest five fractions the deep zone of the articular cartilage. The results were calculated as the mean area-integrated optical density proportional to 1 μm^2 in each zone.

9.4. Uronic acid content

Proteoglycans were quantified as uronic acid. The GAG content of cartilage was calculated as micrograms of uronic acid per milligram of wet weight, as measured with the m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973).

9.5. Electrophoretic analysis of proteoglycans

Cartilage was dissected with a surgical knife from the underlying bone and finely diced with a scalpel blade (unnecessary for rabbit cartilage). After determination of wet weight, proteoglycans were extracted with 500 μl of 4 M guanidinium hydrochloride in 50 mM sodium acetate (pH 5.8), containing inhibitors of bacterial growth and proteases (Lammi et al., 1993). Extraction was continued at 4^{o}C for 24 hours. After the samples were precipitated in ethanol, proteoglycan monomers were characterized by sodium dodecyl sulfate (SDS) agarose gel electrophoresis (Säämänen et al., 1990). The gels were stained with toluidine blue, dried and scanned for densitometry, and exposed to x-ray films for two

weeks. The scanned film and gel densities were analyzed with image analysis software (IP-Lab, Scananalytics, Fairfax, VA, USA) (Lammi et al., 1993).

9.6. Autoradiographic analysis of cartilage zones

For autoradiography, the tissue blocks containing articular cartilage and underlying bone were prepared as described earlier (Parkkinen et al., 1990; Lammi et al., 1993; Suominen et al., 2001), with the exception that no background staining was used. Microscopic analysis of grain area fraction was performed using image analysis. Each microscopic view was divided into ten fields from cartilage surface to the tidemark. The two most superficial fractions represented the superficial zone, the four consecutive fields the intermediate zone, and the four deepest fractions the deep zone. The results are presented as the average grain fraction percentage of the total tissue volume.

9.7. Total incorporation of sulfur-35 sulfate

After metabolic labeling, cartilage was dissected free from underlying bone, weighed, and digested overnight at 60^{O}C with 0.05% proteinase K and 10 mM EDTA. Unincorporated sulfate precursors were removed by gel filtration. Radioactivity in each sample was determined by liquid scintillation counting to study the metabolic activity of the chondrocytes.

9.8. Northern hybridizations

Articular cartilage samples from horses were harvested with a razor blade for pulverization of the tissue (Figure 2A). Both femoral condyles of rabbits were cut for total RNA extraction (Figure 2B). For extraction of total RNA, modification of the guanidinium isothiocyanate method was used (Chirgwin et al., 1979). The samples were frozen, pulverized under liquid nitrogen, homogenized in 4 M guanidinium isothiocyanate, and sedimented through 5.7 M cesium chloride. For Northern analysis, 10-μg aliquots of total RNA were denatured with glyoxal and dimethyl sulfoxide, run on 0.75% agarose gels, and transferred overnight with 20 X SSC onto Pall Biodyne membranes, after which the filters were incubated at 80^{o}C for 2 hours as recommended by the supplier. For Northern hybridizations, cDNA clones were labeled to specific activities of approximately 1x10^{9} cpm/μg using a Random Primed DNA labeling Kit (Boehringer Mannheim, Germany) and ^{32}P dCTP. The hybridizations were performed at 42^{o}C in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, 5 x Denhardt's, and 100 μg/ml sonicated calf thymus DNA. After high-stringency washes, the bound probes were detected and quantified on a Molecular Imager phosphor imager (BioRad, Hercules, CA, USA) and the mRNA signals were corrected for variations in the 28S rRNA levels determined by hybridization. The probes used in Northern hybridizations are listed in Table 2. A cDNA clone for equine type II

collagen mRNA was constructed using the reverse transcription polymerase chain reaction (RT-PCR) technique and total RNA from the equine articular cartilage as a template (detailed description of the RT-PCR technique is given in Study IV).

Table 2. List of cDNA clones used as hybridization probes.

mRNA	Probe	Reference
Proα_1(II) collagen	pECol2α1-1	IV
Proα_1(II) collagen	pKCol2α1-1	Metsäranta et al. 1996
Aggrecan	pHAgg-1	Glumoff et al. 1994
Sox9	pHSox9	Lefebre et al. 1998
28S ribosomal RNA	28S	Iruela-Arispe et al. 1991

10. Statistical analyses

The statistical significance between treated and control groups was analyzed by Wilcoxon's matched-pairs signed-rank test.

RESULTS

1. Clinical signs after RSYN (I, III)

The rabbits showed no signs of discomfort during the experiment. Radiation synovectomy caused severe pain in two horses after treatment of MCP joints. These horses were treated with butorphanol tartrate (Torbugesic®Vet, Fort Dodge Animal Health, Girona, Spain) for nonweight-bearing lameness. After ^{166}Ho FHMA treatment of MTP joints, none of the horses needed treatment for pain. All horses developed circumferential edema of the treated limb. Edema disappeared within 2 weeks, but was replaced by a painful fibrotic thickening around the palmar joint pouch in 4 weeks. Edema made evaluation of effusion difficult, but 5 days after radiation synovectomy aspiration of joint fluid revealed elevated pressure in the joint space. Eight weeks after radiation synovectomy, three horses had forelimb lameness at trot (grade 3 lameness) and two horses had effusion of MCP joints. Control joints showed no pain, effusion, or edema.

2. Autoradiography of rabbits (II)

During the first two days whole-knee autoradiograms of rabbits showed that most of the radioactivity stayed in the joint space (Figure 3A). Two days after ^{166}Ho FHMA injection, some radioactivity was observed inside the bone near the femoral attachment of the cruciate ligament. On the third day, much of the ^{166}Ho FHMA had been phagocytosed by the synoviocytes or had leaked out, i.e. radioactivity was observed between the muscles (Figure 3B). Autoradiograms on the fourth and fifth day showed low levels of radioactivity in the joint space and uneven distribution along the synovium. Autoradiograms of the isolated synovium samples supported uneven incorporation of ^{166}Ho FHMA into the synovium.

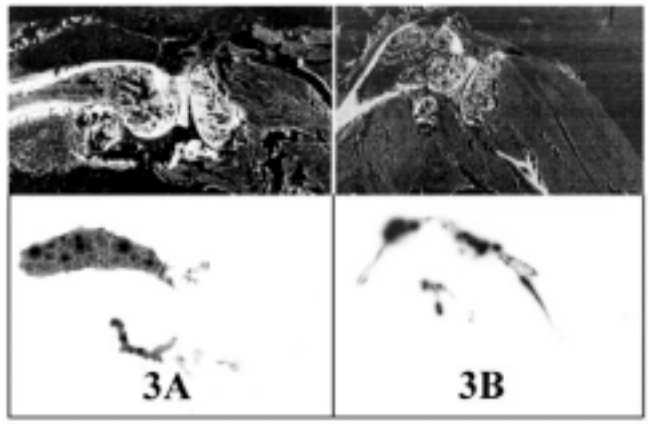

Figure 3. Anatomical sections of whole rabbit knees on cryotape with respective autoradiograms (lower panel) on 1 day (A) and 3 days (B) after intra-articular ^{166}Ho FHMA injection. (A) Patchy distribution of radioactivity in the joint space. (B) Radioactivity between the muscles.

3. Scintigraphy of horses (IV)

In horses, radioactivity was mainly distributed in the proximal plantar joint pouch (on average 82%), and only minor amounts (18%) were detected in other parts of the joints. However, in one MTP joint, radioactivity was more evenly distributed between the proximal plantar joint pouch (58%) and other compartments (40%). The distribution of radioactivity remained the same for 5 days after radiation synovectomy. No obvious extra-articular leakage was detected between days 2 and 5.

4. Arthroscopy of horses (III)

Arthroscopic findings were minimal in the dorsal joint pouch, and cartilage was visually normal. The dorsal aspect of equine fetlock joints was well suited for repeated in vivo measurements of the compressive stiffness of articular cartilage (Figure 4). Compressive testing of articular cartilage showed no difference between reference and follow-up measurements 5 days after RSYN. However, 2 months after RSYN, articular cartilage on the sagittal ridge, but not on the condylar surface, was softer in the treated joint than in the control joint.

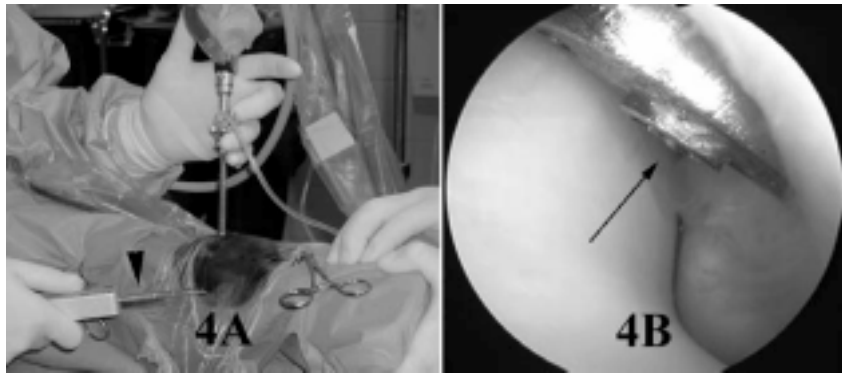

Figure 4. Indentation analysis of equine articular cartilage with an arthroscopic instrument (arrowhead) and an elevated reference plate (arrow). Perioperative view (A) and arthroscopic view (B).

5. Histopathological study of the synovium (I, III)

In rabbits, RSYN caused partial necrosis of the synovial membrane (Figure 5A). In nonnecrotic areas, the synovial lining was 1-2 cells thick, with some polymorphonuclear cells in the subsynovial tissue. In control knees, ^{165}Ho FHMA induced pronounced proliferation of synoviocytes; this was an unusual finding in ^{166}Ho FHMA-treated knees (Figure 5B). In the samples taken 2 months and 1 year after RSYN, regeneration of the synovium, subintimal fibrosis, and subintimal iron deposits in both treated and control knees were typical.

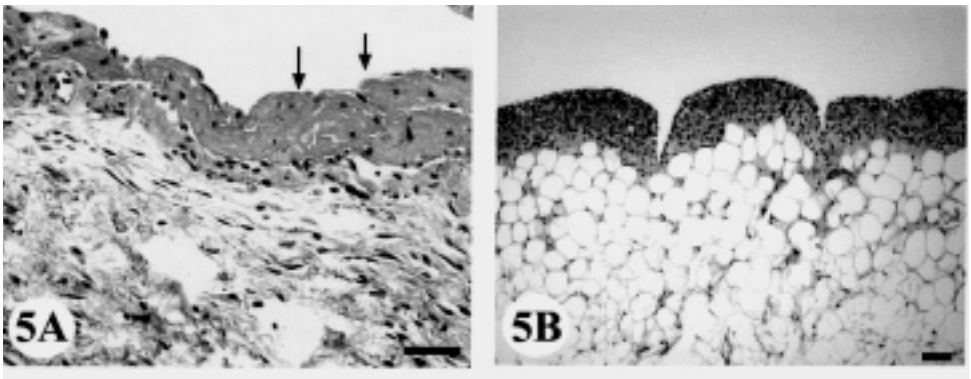

Figure 5. Hematoxylin- and eosin-counterstained sections of lapine synovium 4 days after intra-articular injection of ^{166}Ho FHMA. (A) Necrotic tissue (arrows) on the synovial surface. (B) Control knee injected with nonradioactive ^{165}Ho FHMA; hyperplasia of the synovium. Bar = 50μm.

In horses, histological analyses showed a normal 1- to 2-cell-layer-thick synovial lining in the dorsal joint pouch of all radiosynovectomized joints. Synovial samples from the plantar pouches of MTP joints of horses showed deep multifocal necrosis of the synovium after ^{166}Ho FHMA treatment. Mononuclear cell reaction with congested capillaries and hemorrhage was observed in the subsynovial tissue. Iron-loaded synoviocytes and iron debris were shown in both treated and control joints. By 2 months, large areas of necrotic tissue were still present in the plantar pouches of the MCP joints. Furthermore, large areas of granulation tissue with capillaries, fibroplasia, and mononuclear cell infiltration in the subsynovial tissue were evident. Overall regeneration of the equine synovium was poor 60 days after RSYN despite the presence of granulation tissue.

6. Synovial fluid (I, III, IV)

In rabbits, 4 days after treatment with ^{166}Ho FHMA, white blood cell counts and protein concentration had increased significantly ($p<0.05$) compared with synovial fluid values from control knees. Two months after RSYN, cell counts and protein values were no different from those of control joints.

In horses, synovial fluid protein concentration increased significantly ($p<0.01$) and residual radioactivity was 0.5 MBq/ml in synovial fluid 5 days after RSYN. White blood cell counts were not significantly different from those of control joints. After 2 months, protein concentration had decreased to that of the control joints. Mean GAG concentration was slightly higher in the synovial fluid samples from radiosynovectomized joints, but this increase was not significant compared with samples from control joints.

7. Articular cartilage analyses (II, IV)

In mature rabbits, degenerative changes occurred equally in ^{166}Ho FHMA-treated and control knees. In young rabbits, fibrillation of superficial cartilage layers was observed slightly more often in ^{166}Ho FHMA-treated knees than in control knees. The thickness of articular cartilage, on average 505 μm in mature rabbits and 388 μm in growing rabbits, was unaffected by RSYN. Digital densitometry of GAGs (Table 3) or uronic acid analyses did not show any marked change in composition of articular cartilage; neither was the size of proteoglycans affected by ^{166}Ho FHMA treatment. Both incorporation analysis of tissue samples and autoradiographic analysis of cartilage specimens showed that RSYN had no significant effect on metabolic activity of chondrocytes. In Northern analysis, the levels for pro-α_1(II) collagen mRNA were considerably higher in growing rabbits than in rabbits over 1 year of age (Figure 6). Slightly reduced Sox9 and aggrecan mRNA levels were seen in treated knees of young rabbits 2 months after treatment in comparison with the contralateral control knees (Figure 6). At the 1-year follow-up, no differences were seen in mRNA levels between ^{166}Ho FHMA-treated and control joints.

Table 3. Digital densitometry of safranin-O-stained slides, area-integrated optical density (OD/μm^2) in superficial, middle, and deep cartilage zones.

	Young rabbits			Old rabbits		
	(n=7)	4 days after ^{166}Ho FHMA		(n=6)		
Group	Superficia zone	Intermediate zone	Deep zone	Superficial zone	Intermediate zone	Deep zone
Synovectomy	0.102 ± 0.009[a]	0.135 ± 0.007	0.142 ± 0.006	0.109 ± 0.011	0.155 ± 0.009	0.164 ± 0.011
Control	0.122 ± 0.009	0.150 ± 0.005	0.154 ± 0.005	0.12 ± 0.017	0.154 ± 0.018	0.172 ± 0.014
NC	0.099[b]	0.136	0.147	0.153	0.175	0.184
	(n=8)	2 months after ^{166}Ho FHMA		(n=6)		
Synovectomy	0.119 ± 0.011	0.147 ± 0.009	0.153 ± 0.009	0.127 ± 0.007	0.165 ± 0.005	0.172 ± 0.003
Control	0.128 ± 0.009	0.160 ± 0.006	0.166±0.007	0.118 ± 0.018	0.157 ± 0.014	0.174 ± 0.012
NC	0.125	0.158	0.166	0.153	0.175	0.184
	(n=9)	1 year after	^{166}Ho FHMA	(n=6)		
Synovectomy	0.086 ± 0.010	0.111 ± 0.008	0.121 ± 0.007	0.100 ± 0.012	0.125 ± 0.004	0.132 ± 0.003
Control	0.079 ± 0.007	0.107 ± 0.005	0.119 ± 0.003	0.090 ± 0.014	0.116 ± 0.005	0.126 ± 0.004
NC	0.091	0.109	0.110	0.083	0.104	0.111

NC, Not treated.

[a] Mean ± SEM. [b]Mean values of negative controls.

Data are mean ± standard error of mean (with number of paired observations in parenthesis). The two-tailed nonparametric Wilcoxon's matched-pairs signed-rank test was used for statistical analysis; no significant differences were found between the synovectomy and control groups.

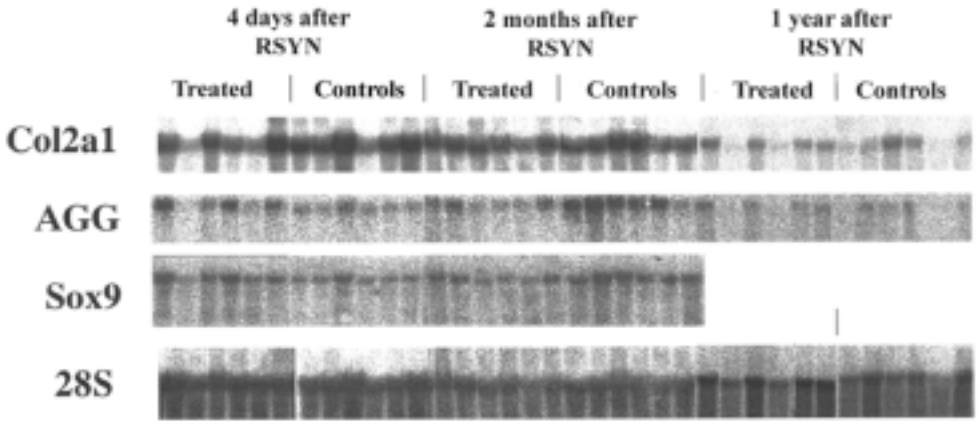

Figure 6. Expression of proα_1(II) collagen, aggrecan, and Sox9 mRNAs in the articular cartilage of young rabbits. The hybridization signals were quantified by phosphor imaging, and the values were normalized against a 28S rRNA signal. Col2a1, proα_1(II) collagen. AGG, aggrecan.

In horses, despite visually normal cartilage, vertical and horizontal clefts were observed in the histological analysis of fetlock joints. The analysis showed no obvious difference in the mean score for cartilage degeneration between treated (1.8; range 1-3) and control (2; range 1-3) joints. Neither was there a significant difference between the mean cartilage thickness of treated and control joints (633 μm vs. 729 μm). Glycosaminoglycan concentration was not altered in any zone of the safranin-O-stained histological samples; nor did uronic acid analysis indicate proteoglycan loss after intra-articular ^{166}Ho FHMA treatment. Migration of proteoglycan bands was not markedly affected by RSYN with ^{166}Ho FHMA, although a slightly decreased average size of proteoglycans was observed in the treated MCP joints. Five days after RSYN, the ^{35}S sulfate incorporation rate into the articular cartilage was not affected, but at the 2-month follow-up the incorporation rate was lower in the radiosynovectomized joints than in the control joints (5.0 ± 1.1 pmol/mg wet weight/hour [mean ± SEM] and 1.8 ± 0.8 pmol/mg wet weight/hour, respectively). The change was not, however, statistically significant. In the autoradiographic analysis of cartilage zones, ^{166}Ho FHMA treatment had no effect on chondrocyte metabolism. Five days after RSYN, mRNA levels for aggrecan were unchanged, but levels for proα_1(II) collagen were decreased in the radiosynovectomized joints compared with the contralateral control joints. However, this change was not significant. At the 2-month follow-up, no differences were seen in the mRNA levels between ^{166}Ho FHMA-treated and control joints.

DISCUSSION

1. General comments on the design of the experiment

Rabbits are often used to test the effects of drugs and various treatments on joints. The most commonly used experimental animal for screening of potential radiosynovectomy agents for human use (Deutsch et al., 1993), the rabbit was also chosen as an experimental animal here. In addition, the horse was deemed a suitable animal since its fetlock joint is nearly the same size as the knee joint of man. Unlike human medicine, veterinary medicine has the benefit of being able to use experimental animals from species that are also clinically relevant. However, horses are expensive and are not readily available for research purposes. The costs can be prohibitive, especially when large numbers of animals need to be tested over extended periods of time.

2. Effects of ^{166}Ho FHMA treatment on the synovium

In both species, treatment with ^{66}Ho FHMA caused multifocal necrosis of the synovium. Living tissue reacts to β radiation by excitation and ionization of the atoms and molecules. Formation of free radicals along with the radiation results in cellular changes both in the nucleus and in the cytoplasm, leading to cell death (Anderson, 1977). The severity of observed synovial abnormalities after RSYN varied in individual rabbits, as has been reported in previous studies (Pavelka et al., 1975; Sledge et al., 1987; Myers et al., 1989). Necrotic synovium could only be evaluated from the knees of mature rabbits since the synovial lining was very fragile in young rabbits. Rabbits and horses had two very different types of synovium; rabbits have an adipose-type subintima in the knee, whereas in the equine fetlock joint it is fibrous-type. This might have had an influence on the severity of symptoms after RSYN. Rabbits did not show any signs of discomfort, while some of the horses had severe lameness and all of the horses had scar tissue formation after ^{166}Ho FHMA treatment. The dose of ^{166}Ho FHMA does not explain the differences in clinical signs since rabbits received high doses of intra-articular radiation relative to the size of the knee.

Absence of hyperplasia in radiosynovectomized joints indicated that a variable number of cells on the morphologically normal synovial lining had lost their ability to divide. The exposure of rapidly dividing cells to intermediate doses of radiation results in a phenomenon known as division delay. Typically, irradiated cells immediately cease division for a period of time that is proportional to the absorbed dose. After this delay, the cells can resume their normal growth patterns (Anderson, 1977). In the present study, intracellular accumulation of iron pigment acted as a direct stimulus to proliferation of the synovium. Effective suppression of inflammatory response using ^{166}Ho FHMA has also been reported in antigen-induced arthritis studies (Chinol et al., 1989, 1990b). Phagocytosing synovial lining cells incite an inflammatory response to any foreign material appearing in the joint

(Henderson, 1988). Here, iron pigment in the subintimal tissue appeared either as a result of intra-articular hemorrhage or iron overload, e.g. intra-articular FHMA (Ghadially, 1983). Both in treated and control joints, the iron pigment persisted in the subintima even 1 year after injection.

3. Side-effects of ^{166}Ho FHMA treatment

Soft tissue swelling and joint effusion lasted for a maximum of 2 weeks in horses. Joint effusion has been described as a common side-effect of RSYN (Lee et al., 2003a). Swelling in humans usually disappears within one month following treatment (Clunie & Fischer, 2003). Intense inflammatory reaction after RSYN has been suggested to reflect effective ablation of the inflammatory tissues (Lee et al., 2003a). However, no correlation was revealed between dose and clinical signs after RSYN with ^{153}Sm M in horses (Yarbrough et al., 2000a). Because only one target dose was chosen for this investigation, the effect of the administered dose on clinical signs is unclear. Simultaneous administration of intra-articular long-acting glucocorticoids has been recommended to reduce radiation-induced inflammatory reaction in humans (Clunie & Fischer, 2003).

Strong β emitter ^{166}Ho penetrated deep into soft tissues, resulting in tissue necrosis, local pain, and restricted range of motion in horses. Compared with the recommended dose for the human knee (124 Gy/knee), 1000 MBq of ^{166}Ho yields a radiation dose of 212 Gy/knee (Zuckerman et al., 1987; Johnson et al., 1995). This dose has been successfully used in man without side-effects (Ofluoglu et al., 2002). Accumulated radioactivity close to sensitive structures in the palmar aspect of fetlock joints may explain the adverse effects in horses. A smaller dose or use of a different radionuclide might cause less harmful side-effects than those observed here. Furthermore, effects on an arthritic equine joint with a thickened synovium could be different from ours.

Increased synovial fluid protein concentration after RSYN indicated radiation-induced, soft-tissue injury and leakage of plasma from the vasculature. The subintimal inflammatory reaction apparent in horses was perhaps due to severe subintimal injury. Subintimal and perivascular clusters of mononuclear cells were still evident 2 months after ^{166}Ho FHMA treatment. The slow regenerative capacity of equine synovium is in accordance with the literature on surgical synovectomy (Theoret et al., 1996; Doyle-Jones et al., 2002). The unaffected white blood cell counts in horses were probably species-related since synovial fluid cell count is a poor indicator of mild joint inflammation in horses (McIlwraith et al., 1979).

4. Distribution of ^{166}Ho FHMA particles

On scintigraphy of horses, ^{166}Ho FHMA accumulated in the palmar joint pouch with abundant synovial tissue. ^{166}Ho FHMA could not have been injected into the palmar subsynovial tissues since a careful injection technique through the lateral collateral sesamoidean ligament was used. Increased synovial pressure might have caused compartmentalization of ^{166}Ho FHMA particles (Strand et al., 1995). Synovial fluid analysis for residual activity suggested that accumulated ^{166}Ho FHMA particles were effectively phagocytosed by the synoviocytes. The distribution was different after ^{153}Sm M synovectomy, in which temporal redistribution into the distal plantar recesses was reported (Yarbrough et al., 2000a). Large-sized ^{153}Sm M particles (20-50 μm) might not be as readily absorbed by the synovium as ^{166}Ho FHMA particles (3-8 μm). The uneven distribution of radioactivity was verified in arthroscopic and histopathological studies. On arthroscopy, the synovium was largely unaffected in the dorsal aspect of the equine joints. Histopathological changes in the palmar/plantar pouch of the fetlock joints were profound compared with those of the dorsal recess of the joint capsule. Uneven RSYN effects concur with other experimental studies describing multifocal necrosis of the synovium after injection with RSYN agents (Sledge et al., 1977; Myers et al., 1989; Yarbrough et al., 2000a). In contrast to findings in the present study, ^{90}Y has previously been shown to be reasonably evenly distributed along the cells of the synovial membrane in rabbits (Ingrand, 1973). Mathematical dosimetric calculations therefore assume an even distribution of radioactivity along the synovium (Johnson & Yanch, 1991). Although ^{166}Ho FHMA particles were well absorbed into the synovium, the uneven distribution renders the validity of the dosimetric calculation for ^{166}Ho FHMA questionable (Johnson & Yanch, 1991). It is not known whether coupling of ^{166}Ho to other carriers, such as hydroxyapatite or chitosan, would have resulted in a more even distribution along the synovium (Song et al., 2001; Unni et al., 2002).

5. Extra-articular leakage of radioactivity

One of the prerequisites of RSYN is that extra-articular leakage and radiation burden to nontargeted organs be minimized. Autoradiograms of rabbit knees showed extra-articular leakage of radioactivity on the third day after ^{166}Ho FHMA treatment. Leakage of a radiopharmaceutical can be caused by a loss of intact particles from the joint, i.e. a loss of enzymatically degraded particles due to reduction below a critical size or a loss of the radioactive label from particles positioned in the synovial pouch (Noble et al., 1983). The count rate performance of the gamma camera was exceeded during the 2 days after ^{166}Ho FHMA treatment of horses, and thus high intra-articular radioactivity could not be recorded accurately. However, scintigraphic analysis showed no obvious extra-articular leakage. Chinol et al. (1990a) reported that FHMA is suitable carrier only for the short-lived radionuclides used in synovectomy. In fact, leakage might be more a function of the short half-life of the radionuclide than the size of the carrier (Deutsch et al., 1993). Previously, use of the same dose of ^{166}Ho FHMA as used in our study of young rabbits caused little extra-articular radiation to the normal organs of adult rabbits (Penttilä et al., 1998).

6. Effects of ^{166}Ho FHMA on the articular cartilage

In adult horses and older rabbits (>1 year old), ^{166}Ho FHMA treatment did not affect the biochemical composition of articular cartilage. In young rabbits, transient derangement of the superficial collagen layer was observed, but the biochemical analysis did not support concomitant change in the size of proteoglycan monomers or loss of proteoglycans. Active matrix synthesis probably protected young animals from permanent cartilage damage.

Stiffness measurements with the indentation device indicated slightly softer equine cartilage after RSYN. Softening of articular cartilage could be associated with loss of proteoglycans from the cartilage (Kempson, 1975). However, the only slight increase in synovial fluid GAGs does not support marked structural changes after ^{166}Ho FHMA treatment (Alwan et al., 1991a), and biochemical analyses failed to confirm loss of proteoglycans from the cartilage. Since the composition and morphology of equine articular cartilage were not markedly affected by ^{166}Ho FHMA treatment, natural degeneration is anticipated to have caused cartilage softening. Further, repeated sequential measurements on the same spots at various times were impossible with the small contact plate. However, it cannot be excluded that ^{166}Ho FHMA treatment provoked cartilage degeneration. The slightly smaller size of aggrecan monomers in the treated joints could reflect this early degenerative process (Vasan, 1980), although the association of reduced size of aggrecan monomers with cartilage degeneration has not been confirmed (Brocklehurst et al., 1984).

Upregulation of cartilage-specific genes, especially of type II collagen, has been observed during early OA (Poole, 1993; Richardson & Dodge, 1997; Aigner et al., 1999; Fehr et al., 2000). In dogs, ^{90}Y synovectomy increased metabolic labeling of proteoglycans, which has been associated with cartilage damage (Carney et al., 1984; Myers et al., 1989). In the present investigation, there was no increased mRNA expression of type II collagen or aggrecan in either species. Autoradiographic grain density was similar in radiosynovectomized and control joints. However, the tissue analysis of chondrocyte metabolism indicated slightly decreased proteoglycan synthesis in equine samples. This discrepancy might be explained by the effect of ^{166}Ho FHMA treatment on chondrocytes being strongest close to the joint margins. Furthermore, large individual variation is present in the ^{35}S incorporation analysis of cartilage samples.

In rabbits, autoradiographic analysis of ^{35}S sulfate-labeled histological sections refuted earlier observations of irradiation-induced chondrocyte injury close to the cartilage surface (Meier-Ruge et al., 1976). The chondrocytes were more likely injured by the chronic arthritis itself than by RSYN (Muller et al., 1975; Hembry et al., 1993). Northern analysis suggested only transient radiation-induced derangements in matrix synthesis of Sox9 and aggrecan mRNAs in knee cartilage of young rabbits 2 months after RSYN. The importance of decreased mRNA expression of aggrecan remains to be determined, as no corresponding changes were seen in proteoglycan or GAG content of the tissue. Sox9 is one of the primary regulators of both aggrecan and the Col2α1 gene (Bi et al., 1999). The reason why downregulated Sox9 mRNA did not result in decreased expression of the

Col2α1 gene was not established here. In horses, the slightly lower mRNA expression of type II collagen 5 days after ^{166}Ho FHMA suggested cytokine-induced downregulation was associated with radiation-induced inflammation (Richardson & Dodge, 1997).

7. General comments on radiosynovectomy with ^{166}Ho FHMA

Our results indicate that ^{166}Ho FHMA could at least temporarily halt the progress of synovial proliferation, thereby decreasing the risk of OA development. MRI studies suggest that the aim of RSYN should be effective long-term suppression of synovial inflammation (Alonso-Ruiz et al., 1998; Pirich et al., 1999). In a clinical study in man, ^{166}Ho FHMA was efficacious in controlling the symptoms of RA (Ofluoglu et al., 2002). However, clinical studies were beyond the scope of this work. Moreover, intra-articular effects of treatment might be different for equine and human joints. Finally, problems associated with dose and with unequal distribution and radiosynovectomy effects on synovial tissues need to be solved before ^{166}Ho FHMA can be implemented for management of synovitis in equine practice.

CONCLUSIONS

Based on the results of these studies, the following conclusions can be made:

(i) Total synovectomy was not achieved even with a high dose of ^{166}Ho FHMA to normal equine and rabbit joints. The aim of ^{166}Ho FHMA treatment should be effective suppression of synovial lining hyperplasia.

(ii) ^{166}Ho FHMA is distributed nonuniformly along the synovium, imparting high concentrations to some areas of the synovium and leaving others untouched. ^{166}Ho FHMA is retained well inside the joint for at least 2 days, and the subsequent risk of extra-articular radiation is small due to the short half-life of ^{166}Ho.

(iii) Articular cartilage was not markedly affected by ^{166}Ho FHMA treatment. Mild cartilage fibrillation and temporary downregulation of Sox9 and aggrecan genes had no permanent consequences on young rabbit cartilage. In adult horses, transient down regulation of type II collagen synthesis and a slight reduction in the size of aggrecan monomers had no obvious effect on biochemical or morphological properties of articular cartilage.

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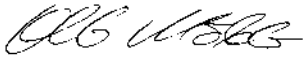

Olli Mäkelä

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